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Molecular, physiological and morphological analysis of waterlogging tolerance in clonal genotypes of *Theobroma cacao* L.

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Summary In soil, anoxia conditions generated by waterlogging induce changes in genetic, morphological and physiological processes, altering the growth and development of plants. Mass propagation of cacao (*Theobroma cacao* L.) plantlets (clones) is affected by waterlogging caused by heavy rains and irrigation methods used to induce rooting. An experiment was undertaken to assess the effects of a 45-day flooding (anoxia) on physiological and morphological traits of 35 elite cacao genotypes, aiming at potentially identifying those with greater tolerance to flooding of the growth substrate. Eighteen fluorochrome-labeled microsatellite (SSR) primer pairs were used to assess genetic variability among clones, with 248 alleles being amplified and used to calculate similarity coefficients. The resulting dendrogram indicated the presence of four major groups, in which two represented 60% and 31% of the genotypes tested. A general trend toward high levels of heterozygosity was also found for physiological and morphological traits. The survival index (IS) for flood tolerance observed varied from 30 to 96%. Clones TSA-654, TSA-656, TSA-792, CA-1.4, CEPEC-2009 and PH-17 showed an IS value above 94%, whereas CEPEC-2010, CEPEC-2002, CA-7.1 and VB-903 clones were those mostly affected by waterlogging, with IS value below 56%. All genotypes displayed lenticel and adventitious root formation in response to waterlogging, although with different intensities. To determine whether patterns of physiological response could be associated with tolerance to anoxia, a similarity-grouping analysis was performed using the ratio between waterlogged and control values obtained for a series of physiological variables assessed. No specific pattern of physiological and morphological responses to waterlogging was strictly associated with survival of plantlets. However, results revealed by the dendrogram suggest that absence of leaf chlorosis may be a proper trait to indicate cacao clones with higher survival rates under flooding conditions. Consequences of these findings are discussed in the context of developing improved strategies for mass production of clones from elite cacao genotypes.

Keywords: anoxia stress, cacao, genetic variability, photosynthesis, SSR markers.

Introduction

Cacao (*Theobroma cacao* L.) is considered one of the most important perennial crops in the world due to its economical relevance for chocolate and cosmetics industries (Almeida and Valle 2007). Brazil is the world's fifth largest producer of cocoa beans, with investments of ~US$ 1.35 billion that provide direct and indirect jobs to ~3 million people. The southeastern Bahia State is the main cocoa-producing region of the country, with this crop planted on more than 700,000 ha, an area corresponding to ~29,000 farms (Souza and Dias 2001). The outbreak of the witches' broom disease in 1989 (Pereira et al. 1989), caused by the fungus *Moniliophthora perniciosa* (Aime and Phillips-Mora, 2005), had severely affected social, economical and ecological settings of this region, leading to an urgent development of strategies to control the disease.

Since the 1990s, cloning of genotypes resistant to this disease and their distribution to farmers has been the main approach for the rehabilitation of the cocoa-based economy (Souza and Dias 2001). Newly developed witches' broom-resistant clones have been recommended by the country's Executive Commission for Development of the Cacao Crop (CEPLAC), not only to establish more uniform and productive plantations but also to replace susceptible canopies of old crops. These improved genotypes are maintained mostly at the Cacao Research Centre (CEPEC—http://www.cepec.gov.br) of CEPLAC, and, as this is an alogamous species,

cuttings from plagiotropic branches are used for the mass production of clonal plantlets for distribution to cacao growers. In order to obtain an appropriate adventitious rooting for these cuttings, the chosen propagation scheme requires that leaf moisture be kept at 100%, with air relative humidity around 60–70% for the first 45 to 60 days of growth. These conditions are achieved by a regime of intermittent water atomization (microaspersion) over the plantlets (Palacios and Monteiro 2000). However, periods of heavy rainfall in addition to microaspersion create waterlogging effects, which lead to anoxia within the growth substrate. For certain genotypes, such conditions can cause the death of up to 70% of the plantlets, thereby preventing the proper multiplication of their clones.

Conditions of anoxia cause physical, chemical and biochemical changes in the substrate. Waterlogging causes exclusion of air from soil that decreases oxygen levels, thereby creating a reducing environment. Oxygen is rapidly consumed by soil microorganisms and plant root respiration, which leads to various degrees of molecular oxygen depletion (*hypoxia*) or absence (*anoxia*). In these circumstances, iron is reduced to Fe^{2+} and manganese to Mn^{2+}, which can be toxic to plants (Marschner 1995, Fageria et al. 2002). Reducing conditions also enhance the accumulation of other potentially phytotoxic compounds from the anaerobic metabolism of microorganisms and plants (Pezeshki 2001, Grichko and Glick 2001). Moreover, anaerobic respiration of the root system decreases the amount of energy produced, interfering with several housekeeping biosynthetic routes such as chlorophyll synthesis (Dennis et al. 2000). Waterlogged plants show distinct genetic, physiological and morphological responses that include stomatal closure followed by reduced photosynthesis, altered biomass production, altered gene expression with synthesis of anaerobic proteins, development of aerenchyma and hypertrophied lenticels, etc. (Sachs et al. 1980, Kozlowski 1997).

Interactions of morphological and physiological adaptations with induced gene expression are reported as tolerance responses to waterlogging in certain woody plants (Kozlowski 2002). Therefore, high levels of genetic diversity are required in a given population to allow selection procedures towards this trait. Especially for perennial crops, the use of molecular markers has been largely employed for the assessment of genetic variability because it grants the convenience of analyzing a great number of polymorphisms at the DNA level, in a short timeframe and without environmental interference (Pires et al. 2000, Risterucci et al. 2000*a*, 2000*b*).

Considering that upper Amazonia, with its humid tropical climate, is the center of origin and dispersion of cacao (Warren 1993), it is conceivable that genetic variability exists for waterlogging tolerance in this species. In our earlier studies, interclonal variation regarding tolerance to O_2 deficiency in the substrate has in fact been observed during multiplication of *T. cacao* cuttings, as different clones display varying survival percentages under waterlogging conditions. Hence, the objectives of this work were (i) to verify the level of genetic variability among 35 *T. cacao* clones that are highly productive and resistant to witches' broom disease, (ii) to test the hypothesis that tolerance to anoxia in cacao clones can be identified by combining physiological and morphological variables as response to waterlogging and (iii) to obtain critical information to optimize multiplication of the recommended cacao genotypes on a clone-specific basis, by a more efficient management of irrigation and rooting of the branch cuttings.

Materials and methods

Plant material and growth conditions

Thirty-five *T. cacao* clonal genotypes regarded as resistant to witches' broom disease were assessed. The clones, labeled as BE-07; CA-1.4 and -7.1; CCN-10 and -51; CEPEC-42, -2001, -2002, -2004, -2005, -2006, -2007, -2008, -2009, -2010 and -2011; CP-06, -49 and -53; HW-25; PH-15, -16, -17 and -92; PS-13.19; SJ-02; TSA-654, -656, -792 and -774; TSH-1188; VB-276, -679, -902 and -903, were all obtained from the cacao germplasm collection of CEPEC/CEPLAC (Ilhéus-BA, Brazil) and multiplied at 'Instituto Biofábrica de Cacau' (IBC, Banco do Pedro, Ilhéus-BA), an institution funded by the Bahia State government. The plantlets for the experiments were obtained by rooting ~16-cm-long stem cuttings from plagiotropic branches at the beginning of secondary growth, containing the apical bud, three auxiliary buds and three leaves. The bottoms of the cuttings (~3 cm) were dipped into chemically inert talcum powder containing indol-3-butyric acid (IBA) at 4 g kg^{-1}. Afterwards, each cutting was transferred to a 288-cm^3 tube-like, black plastic pot containing organic substrate (turf + grinded *Pinus* sp. barks and grinded coconut fiber at 1:1 ratio) enriched with macro and micronutrients, according to the recommendations for cacao plants (Souza 2007). The planted pots were left at a screenhouse with 50% sunlight cover and irrigated by microaspersion. The waterlogging treatment was performed for 45 days by placing 50 planted tube-like pots of each clone into plastic 30-l trays filled with water up to 20 mm above the substrate level. Fifty control pots for each clone were placed in trays of the same type, perforated at the bottom to allow drainage of the irrigation-water excess.

DNA extraction and SSR analysis

Total DNA was extracted from fresh leaf tissue of clonal plantlets between 3 and 4 months after rooting, using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990), modified by Corrêa et al. (1999). Microsatellite (SSR) markers were used for analysis of interclonal genetic diversity, using 18 fluorochrome-labeled primer pairs as described in Table 1. Amplification reactions were performed in a total volume of 13 µl per sample, containing recommended buffer, 50 mmol l^{-1} $MgCl_2$, 2.5 mmol l^{-1} each deoxyribonucleotide triphosphate (dNTP), 10 µmol l^{-1} primers, 2.5 mg μl^{-1} bovine serum albumin, 5 U μl^{-1} *Taq* poly-

Table 1. Description of SSR markers[1] used in the study

Accession no.[2]	SSR marker	Annealing temp. (°C)	Size (bp)	Motif
AJ271827	mTcCIR 35	46	235	$(GT)_{11}$
AJ271942	mTcCIR 37	46	150	$(GT)_{15}$
AJ271944	mTcCIR 42	46	232	$(CA)_{21}$
AJ271945	mTcCIR 43	46	206	$(TG)_5(TA)$
AJ271946	mTcCIR 44	51	178	$(GT)_{10}$
AJ271953	mTcCIR 54	46	165	$(CA)_{15}$
AJ271956	mTcCIR 57	46	253	$(AC)_{13}$
Y16883	mTcCIR 1	51	143	$(CT)_{14}$
Y16977	mTcCIR 3	46	249	$(CT)_{20}(TA)_{21}$
Y16978	mTcCIR 2	51	254	$(GA)_3N_5(AG)_2GG(AG)_4$
Y16980	mTcCIR 6	46	231	$(TG)_7$ $(GA)_{13}$
Y16981	mTcCIR 7	51	160	$(GA)_{11}$
Y16982	mTcCIR 8	46	301	$(TC)_5TT(TC)_{17}TTT(CT)_4$
Y16983	mTcCIR 9	51	274	$(CT)_8N_{15}(CT)_5N_9(TC)_{10}$
Y16984	mTcCIR 10	46	208	$(TG)_{13}$
Y16985	mTcCIR 11	46	298	$(TC)_{13}$
Y16986	mTcCIR 12	46	188	$(CATA)_4N_{18}(TG)_6$
Y16987	mTcCIR 13	46	258	$(AG)_{13}$

[1]Lanaud et al. 1999; Risterucci et al. 2000*a*, 2000*b*.
[2]Genbank (www.ncbi.nlm.nih.gov).

merase and 30 ng DNA as template. The cycling conditions were 30 cycles of 2 min at 96 °C, 1 min at 46 °C or 51 °C (according to primers used), and 1 min at 72 °C, with a final extension step of 7 min at 72 °C; after completion, temperature was held at 15 °C. The amplification products were separated by 4% polyacrylamide gel electrophoresis run at an ABI-377 automated sequencing system (Applied Biosystems), according to manufacturer's recommendations. The study and characterization of the amplified SSR markers were performed by using the accompanying software 'Genescan' and 'Genotyper'.

Genetics and statistical analyses of molecular data

The amplification data from the SSR markers were scored (marker presence = 1, absence = 0, lost information = 9) and converted into a numerical matrix from which the coefficients of genetic similarity were calculated and the grouping analysis performed for the cacao genotypes. Coefficients of genetic similarity were calculated by the arithmetical complement of Dice's similarity coefficient (Nei and Li 1979, Corrêa et al. 1999). The Unweighted Pair Group with Arithmetic Mean (UPGMA) method was used as a grouping criterion for the calculated matrix of genetic similarity among genotypes. The level of heterozygosis was assessed as the ratio between the number of heterozygous loci and the total number of analyzed loci.

Photosynthetic parameters

Photosynthetic characteristics were evaluated 45 days after starting waterlogging treatments. Leaf gas exchange measurements were done on matured leaves of eight plantlets for each cacao clone (four waterlogged and four controls), always between 7:30 AM and 1:30 PM, using a portable photosynthesis system Li-6400 (Li-Cor, Nebraska, USA) equipped with an artificial light source 6400-02B RedBlue. The equipment was adjusted to a photosynthetic photon flux density (irradiance) of 800 µmol photons m^{-2} s^{-1}, i.e., above the light saturation irradiance needed for cacao photosynthesis. The net photosynthetic rate by unit of leaf area (A), stomatal conductance to water vapor (gs) and leaf transpiration rate (E) were estimated from atmospheric CO_2 (Ca) and air humidity values measured inside the chamber. The intercellular CO_2 concentration (Ci) was automatically estimated by the equipment based upon the values of A, gs and E (von Caemmerer and Farquhar 1981). Other variables assessed were the Ci/Ca ratio, intrinsic (A/gs) and instantaneous water use efficiency (A/E or WUE) at the level of irradiance described above. To assess the chlorophyll fluorescence in dark-acclimated leaves, a clip was set for 30 min in each leaf for reflection of solar radiation, decrease of leaf temperature and oxidation of the whole photosynthetic electron transport system. Using the fluorescence meter accessory of the photosynthesis system LI6400, the dark parameters minimal (F_o), maximal (F_m) and variable (F_v) fluorescences as well as the maximum quantum yield (F_v/F_m) of photosystem II (PS II) were measured and recorded (Maxwell and Johnson 2000).

Growth parameters

Evaluation of the survival index (IS) was done after 60 days of planting, when all cuttings had rooted appropriately. To obtain the partial and total dry biomasses, 16 plantlets from each clone (eight waterlogged and eight controls) were collected at the end of the experiment, subdivided into roots, stems and leaves, placed separately in paper bags and set immediately to dry at 75 °C until constant weight. Total leaf

area (LA) was measured by a Li-3100 apparatus (Li-Cor, NE, USA), and the number of leaves (NL) was counted per plantlet. From these procedures and according to methods described by Radford (1967) and Richards (1969), the following variables were then assessed: root biomass (RB), leaf biomass (LB), stem biomass (SB), individual leaf area (ILA = LA/NL), specific leaf biomass (SLB = LB/LA) and roots to shoots biomass ratio [R/S = RB/(LB + SB)].

Analysis of similarity based on physiological and morphological variables

Ratios between waterlogged and control treatments per clone were taken based on the values obtained for the variables *A*, gs, *E*, Ci/Ca, WUE, *A*/gs, F_o, F_m, F_v/F_m, LB, SB, RB, TB (total biomass), R/S, NL, LA, ILA and SLB and used to calculate the distance among materials and to proceed with group analysis. The matrix of physiological similarity among genotypes was calculated based upon the Euclidian distance algorithm, using the UPGMA method as the grouping criterion.

Statistical analyses

A completely randomized experimental design was employed, with treatments being 35 clones × 2 water regimes (waterlogged and control). Each of the 70 treatments had a single plantlet as the experimental unit, with 50 replicates (a total of 100 plantlets assessed per clone). Results were subjected to analysis of variance (ANOVA), with mean comparisons between waterlogged and control treatments done by the Student's *t*-test ($P < 0.05$).

Results

Waterlogging effects on the survival index of clonal cacao plantlets

In order to assess the magnitude of the flooded-substrate effect on the survival index of clonal plantlets, previously collected data from 60-day-old rooted cuttings of 14 clones obtained for three consecutive years of production at the IBC were analyzed (Figure 1). A clear inverse association between clone survival and monthly rainfall was observed; for instance, the overall decrease in the average IS values of the clones observed for the months of February through August (Figure 1A) essentially corresponded to periods of higher rainfall in the region (Figure 1B). In this period, more than 25% of the clones displayed very low IS values, with some of them close to zero, indicating an almost complete death of plantlets (Figure 1B).

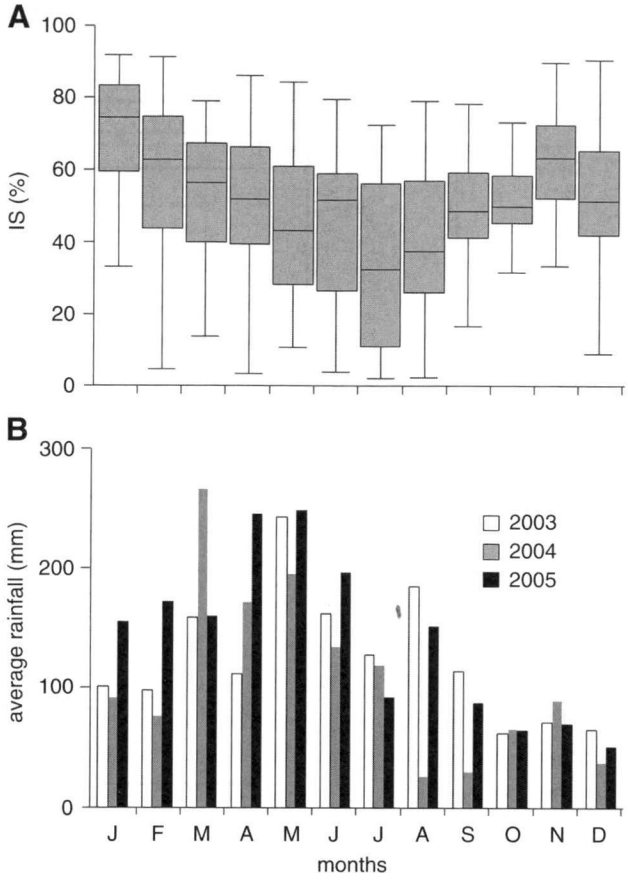

Figure 1. Relationship between variation in the rainfall intensity and survival index (IS) of cacao clones in three subsequent years. (A) Standard box plot of the monthly average IS per clone obtained at the IBC institute for rooted stem cuttings of 14 genotypes (CA-1.4; CCN-10 and -51; CEPEC-2002, -2004, -2005, -2006, -2007 and -2008; CP-06, PH-16, PS-13.19, TSA-792 and TSH-1188); gray boxes represent 50% of values, with the intersecting horizontal line indicating the median; data obtained from a total of 420,000 clonal plantlets. (B) Monthly average rainfall of years 2003, 2004 and 2005 at the 'Banco do Pedro' district (Ilhéus-BA), where the IBC institute is located.

Analysis of genetic diversity among cacao clones

Microsatellite molecular markers (SSR) were employed to investigate the level of interclonal genetic diversity among the main cacao genotypes recommended for planting in the southeastern region of Bahia. The 18 SSR-primer pairs used (Lanaud et al. 1999, Risterucci et al. 2000) revealed a total of 248 alleles, with an average of 13.8 alleles per locus. The shortest amplified fragment (allele) obtained was 125 bp and the largest 366 bp (Table 1). The similarity analysis based upon the presence or absence of alleles indicated the existence of genetic variability among the 35 cacao clones tested (Figure 2). Using a similarity cutoff of 25%, four major groups were identified: one containing a single genotype (CCN-51), another with TSA-654 and -656, and two others containing 60% and 31% of the remaining clones. The maximum level of similarity found among genotypes was 72% for the clones CEPEC-2002 and PH-17. The four clones showing no chlorosis under waterlogging conditions (see further below) were grouped apart from each other, showing less than 35% similarity (Figure 2). In general, high levels of heterozygosis were observed, with ~55% of the tested

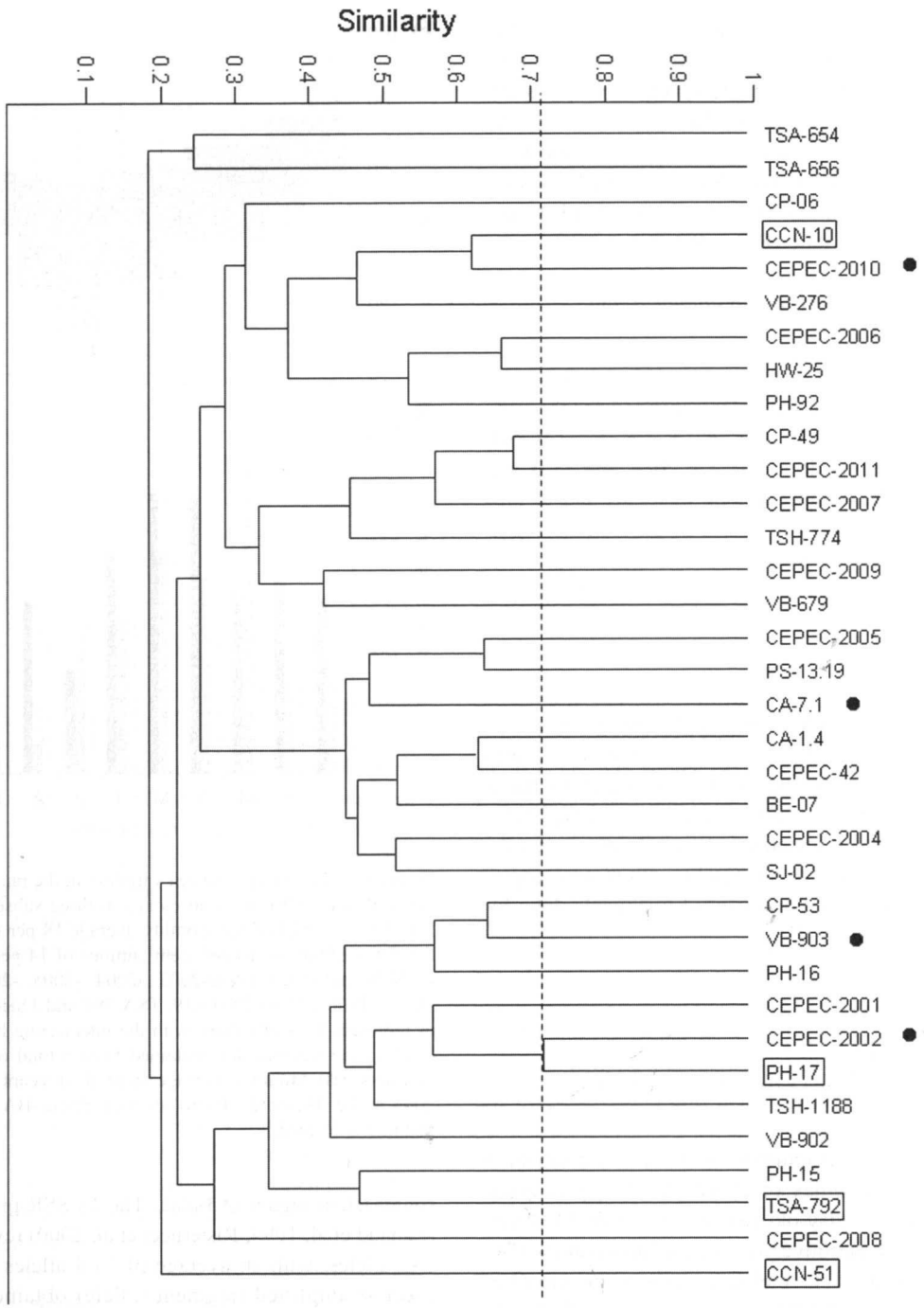

Figure 2. Grouping analysis of 35 cacao clones based upon the arithmetical complement of Dice's coefficient of similarity, using SSR markers and the UPGMA method. The maximum level of 72% similarity is indicated by a vertical dashed line. Clones indicated on right by a box or a black dot showed no chlorosis or the lowest IS values, respectively, after waterlogging treatment (see Table 2).

genotypes showing more than 50% of their loci as being heterozygous. The TSH-1188 clone was the most heterozygous (>90% of loci), whereas CA-7.1 was the least (<30% of loci).

Analysis of physiological and morphological responses of cacao clones to waterlogging

Based upon the interclonal genetic diversity observed and aiming at the possible identification of cacao genotypes more tolerant to waterlogging, the effects of anoxia established by flooding the growth substrate were investigated. Out of 35 clones evaluated, 27 (77.1%) showed IS values above 80%, with TSA-792, -654, -656 and CA-1.4 showing the highest IS value (96%). The lowest IS value (30%) was observed for the clone VB-903 (Table 2). Typical symptoms of anoxia stresses such as leaf chlorosis and formation of lenticels and adventitious roots were also evaluated. With the exception of CCN-

10 and -51, PH-17 and TSA-792, the other clones displayed different chlorosis intensities. Taking all three waterlogging-related stress symptoms into consideration, these four clones presented the least affected phenotypes with high IS values, ranging from 82 to 96% (Table 2). A trend towards negative association between stress symptoms and survivability was noticed (Table 2), although with some exceptions. For instance, some clones showed higher intensities for all symptoms but gave high IS values (e.g., CA-1.4, CP-06, CEPEC-2008 and -06); conversely, clones with lower IS values did not necessarily show high levels of stress symptoms (e.g., TSH-774 and CEPEC 2002).

A more detailed assessment of genotypic responses to anoxia conditions was performed in terms of photosynthetic parameters. After 45 days of waterlogging, most of the clones showed significant reductions ($P < 0.05$) for photosynthetic rates (A) and stomatal conductances to water vapor (gs) in relation to the controls, with the exception of CCN-10, PH-17 and TSA-792 for both variables, CA-7.1 and SJ-02 for A and CEPEC-2002 for gs (Table 3). Maximum values for the control plantlets were observed for CEPEC-2005 (A = 8.40 μmol CO_2 m^{-2} s^{-1}) and CEPEC-2004 (gs = 0.14 mol H_2O m^{-2} s^{-1}), whereas for the waterlogged treatments they were for CCN-10 (A = 7.43 μmol CO_2 m^{-2} s^{-1}) and CEPEC-2002 (gs = 0.08 mol H_2O m^{-2} s^{-1}). On the other hand, the minimum values of A and gs were 3.83 μmol CO_2 m^{-2} s^{-1} (CEPEC-2011) and 0.05 mol H_2O m^{-2} s^{-1} (CEPEC-2009), respectively, for the control treatments and 0.12 μmol CO_2 m^{-2} s^{-1} (CA-1.4) and 0.01 mol H_2O m^{-2} s^{-1} (CEPEC-2010) for the waterlogged treatments. The control plantlets for all clones showed values close to 0.8 for the maximum quantum yield of PS II (F_v/F_m). Treatments were significantly different ($P < 0.05$) for 20 out of the 35 clones (57.1%) in terms of minimal (F_o) and maximal (F_m) fluorescences as well as for the maximum quantum yield, with a tendency of higher F_o and lower F_m and F_v/F_m values for the waterlogged plantlets (Table 3). The minimal F_o and F_m values observed for the control plantlets were 278.6 (PH-17) and 1241.75 (VB-903), respectively, whereas for the waterlogged ones they were 294.10 and 620.03 (both for CEPEC-2008); the maximal F_o and F_m values were 335.73 (CEPEC-2009) and 1578.70 (HW-25), respectively, for the control plantlets, and 507.55 (CEPEC-2001) and 1714.48 (CEPEC-2004) for the waterlogged.

Imposed anoxia conditions on different genotypes significantly influenced leaf biomass (LB) for 20% of the clones, root biomass (RB) for 49% of the clones and the pattern of biomass allocation (R/S ratio) for 37% of the clones (Table 3). The lowest LB values were found for the waterlogged treatments of CEPEC-2007, -2010 and VB-903 clones, with decreases of 43, 32 and 35%, respectively, in relation to their controls. Waterlogged treatments for CEPEC-2005, HW-25, TSH-774 and PH-17 genotypes showed the largest differences in relation to their controls for the RB and R/S variables, with reductions in a range of 64–60% and of 62–55%, respectively. Waterlogging significantly reduced leaf number (NL) for six (17%) and leaf area (LA) for 10 (29%) clones; decreases in NL ranged from 31 to 20% when compared to the controls. The four clones that gave the largest decreases in LA (31–35%) were CEPEC-2008, -2010, CP-53 and PH-16. The maximum LA and NL values observed were 5.9 $m^2 \times 10^{-2}$ (CEPEC-2010) and 11.4 (VB-903), respectively, for the control and 5.0 $m^2 \times 10^{-2}$ (TSA-792) and 10.8 (CP-06) for the waterlogged, whereas the minimum were 2.7 $m^2 \times 10^{-2}$ (SJ-02) and 6.0 (PH-92) for the control, and 2.4 $m^2 \times 10^{-2}$ (CP-53) and 5.6 (CEPEC-2008) for the waterlogged, respectively.

The four clones with no symptoms of chlorosis (CCN-10, -51, PH-17 and TSA-792, Table 2) have also shown no more than three physiological and morphological variables with statistical differences between waterlogged and control plants. Such a criterion, however, was not strictly associated with a higher IS (Table 3). For instance, the CA-7.1 and CEPEC-2002 genotypes displayed three or fewer variables

Table 2. Morphological responses[1] and survival index of 35 cacao clones after 45 days of growth under waterlogging conditions of the substrate

Clone	Chlorosis	Lenticels	Adventitious roots	IS (%)
CCN-51	0	+	+	82
PH-17	0	+	+	94
TSA-792	0	+	++	96
CCN-10	0	++	+	82
CEPEC-2005	+	+	+	80
CEPEC-2006	+	+	+	82
PH-92	+	+	+	90
TSA-656	+	+	+	96
TSH-774	+	+	+	72
BE-07	+	+	++	88
CEPEC-2007	+	++	+	82
PH-16	+	++	++	88
PS-13.19	+	++	++	64
VB-679	+	++	++	84
CEPEC-2010	+	++	+++	54
CP-49	+	++	+++	84
CA-1.4	+	+++	++	96
SJ-02	+	+++	++	82
CP-06	+	+++	+++	84
HW-25	+	+++	+++	76
CA-7.1	+	+++	+++	44
CEPEC-2001	++	+	+	88
TSH-1188	++	+	+	86
VB-276	++	+	+	86
CP-53	++	+	++	76
TSA-654	++	+	++	96
CEPEC-2002	++	++	++	56
PH-15	++	+++	++	66
VB-902	++	+++	+++	68
CEPEC-2008	+++	++	+	86
CEPEC-2009	+++	+	+	94
CEPEC-2011	+++	+	+	84
CEPEC-42	+++	+	++	86
VB-903	+++	++	+	30

[1] An arbitrary scale was established to assess presence and intensity of the morphological characteristics studied, in which '0' = absence, '+' = low, '++' = medium and '+++' = high.

Table 3. Difference (Δ) in values between control and waterlogged plantlets for physiological and morphological variables[1] assessed on 35 cacao clones

Clone	*A*	*gs*	F_o	F_m	F_v/F_m	LB	RB	R/S	NL	LA	#Var[2]
BE-07	1.27**	0.03**	−72.38	359.08*	0.16**	0.18	0.24	0.05	0.25	−0.01	4
CA-1.4	5.74**	0.05**	−8.60	−59.05	0.00	0.10	0.80**	0.15**	2.50	−0.06	4
CA-7.1	3.69	0.07**	−19.10	448.20**	0.14*	0.26	0.12	0.01	0.75	−0.02	3
CCN-10	−1.15	0.03	−54.85	−290.82	0.00	0.29	0.27	0.04	0.88	−0.07	0
CCN-51	2.27*	0.03*	−35.23	171.60	0.06	0.14	0.16*	0.02	0.13	0.02	3
CEPEC-2001	4.96**	0.06**	−213.88*	263.33	0.25*	0.39	0.15	0.01	0.25	0.04	4
CEPEC-2002	2.53**	0.00	−65.68	215.98	0.09	0.58	0.22*	0.03	1.00	0.02	2
CEPEC-2004	5.80**	0.10**	−61.33*	−240.80	0.01	0.96*	0.70**	0.07*	2.13*	−0.02**	8
CEPEC-2005	7.46**	0.10**	−78.13	545.05*	0.23*	0.11	1.40**	0.32**	2.75	0.02*	7
CEPEC-2006	5.82**	0.08**	−137.93*	403.60*	0.22*	0.21	0.38*	0.07**	1.50	0.02*	8
CEPEC-2007	4.84**	0.11**	−2.73	391.08*	0.09*	1.11**	0.01	−0.06	0.63	0.02	5
CEPEC-2008	4.50**	0.05*	8.57	792.58**	0.26**	0.10	0.14	−0.01	3.13**	−0.07**	6
CEPEC-2009	2.46*	0.04	−166.88*	211.75	0.21	0.21	0.67**	0.13**	3.13	−0.14	4
CEPEC-2010	4.37**	0.05**	−92.58*	654.50**	0.33**	0.98*	0.69*	0.04	3.88*	−0.02*	9
CEPEC-2011	1.99*	0.05**	−58.08*	144.58	0.09*	0.85**	0.32	0.02	2.13	−0.05	5
CEPEC-42	3.38**	0.04**	7.45	755.50**	0.23*	0.78*	0.49**	0.06	1.38	−0.01	6
CP-06	1.94**	0.06**	−52.38	−123.33	0.03	0.11	0.48*	0.09*	−0.13	0.02	4
CP-49	4.49**	0.06*	−44.48	45.45	0.05	0.16	0.33	0.06	−1.13	0.07	2
CP-53	4.81**	0.07*	−190.15*	258.93*	0.23**	0.50*	0.02	−0.03	1.88	0.05**	7
HW-25	4.84**	0.06*	−148.90**	658.45**	0.31**	0.06	0.88**	0.17**	0.50	−0.01	7
PH-15	5.01**	0.08**	−2.50	736.13*	0.24**	0.01	0.01	−0.01	0.75	0.03	4
PH-16	2.62*	0.05*	−14.45	257.28	0.08	0.48	0.68*	0.11**	0.00	0.16**	5
PH-17	−0.41	0.01	−27.05	−38.93	0.01	0.06	0.94**	0.21**	−0.75	0.11	2
PH-92	3.83**	0.06**	−142.70	284.95**	0.17*	0.03	0.60**	0.14**	−0.63	−0.02	6
PS-13.19	4.25*	0.06*	−42.83	−55.93	0.05	0.06	0.34*	0.09*	1.88*	−0.08	5
SJ-02	3.14	0.05**	−144.80	244.05	0.19*	0.17	0.32*	0.08*	0.75	−0.07	4
TSA-654	5.36**	0.07*	−82.95	293.93	0.15**	0.09	0.62	0.13	0.50	0.03	3
TSA-656	5.72**	0.08**	−74.45**	634.18**	0.28*	0.49	0.02	−0.07	0.63	0.12*	6
TSA-792	1.32	0.02	−72.03	38.85	0.06	0.31	0.14	0.02	0.25	0.02	0
TSH-1188	4.32**	0.09**	−83.23*	315.63	0.14*	0.26	0.06	−0.01	1.00	−0.04	4
TSH-774	5.68**	0.05**	−131.80**	544.68*	0.33*	0.25	2.15**	0.40**	0.75	−0.02	7
VB-276	5.49**	0.05**	−98.35	534.28*	0.22**	0.06	0.02	0.00	0.50	−0.07	4
VB-679	5.02**	0.08*	−70.88	−66.97	0.04	0.10	0.01	0.00	0.00	0.00	2
VB-902	4.86**	0.07**	−96.75	61.60	0.09	0.46	0.45	0.05	1.75*	−0.02*	4
VB-903	3.92**	0.05**	−46.35	350.75*	0.15	0.97*	0.66	−0.02	2.88**	0.06**	6

Statistical significance for the differences between control and waterlogged treatments is indicated as follows: $*P < 0.05$; $**P < 0.01$.

[1] *A*, net photosynthetic rate per unit of leaf area (μmol CO_2 m^{-2} s^{-1}); gs, stomatal conductance to water vapor (mol H_2O $m^{-2}s^{-1}$); F_0, initial fluorescence (relative unit); F_m, maximal fluorescence (relative unit); F_v/F_m, maximum quantum yield; LB, leaf biomass (g); RB, root biomass (g); *R/S*, roots to shoots biomass ratio; NL, number of leaves; LA, leaf area (m^2 $plantlet^{-1}$).

[2] Number of variables showing statistically significant difference between control and waterlogged plantlets for each clone.

as significantly different between waterlogged and control treatments, but their IS values were among the lowest observed, i.e., 56 and 44%, respectively; by the same token, the TSA-656 and PH-92 clones presented six out of 10 variables with statistical differences between treatments, and yet their IS values were >90% (Tables 2 and 3).

Diversity of physiological responses to waterlogging as a grouping criterion

To determine whether or not physiological and morphological traits could provide suitable criteria for identification of tolerant clones to anoxia of the growth substrate, a grouping analysis of the cacao genotypes under study was performed based on those variables shown in Table 3. The ratio between the values obtained from waterlogged and control treatments for the 35 clones were used to build a similarity matrix and dendrogram (Figure 3). The results indicated the existence of physiological variability among the genotypes, and, based on a 70% similarity-value cutoff (Euclidian distance of −0.3), six main groups of clones were observed: two groups with a single clone (BE-07 and CCN-10 groups), one group with two clones (CA-1.4 and CEPEC-2006), two groups with three clones (one with CEPEC-2002, -42 and CP-49, another with CEPEC-2010, TSA-656 and TSH-774) and the largest sixth group containing 71% of the evaluated genotypes. Strict association between grouping based on physiological variables and higher or lower tolerance to waterlogging was not found, since the four clones with IS values in the lowest 30–56% range (i.e., VB-903, CA-7.1, CEPEC-2002

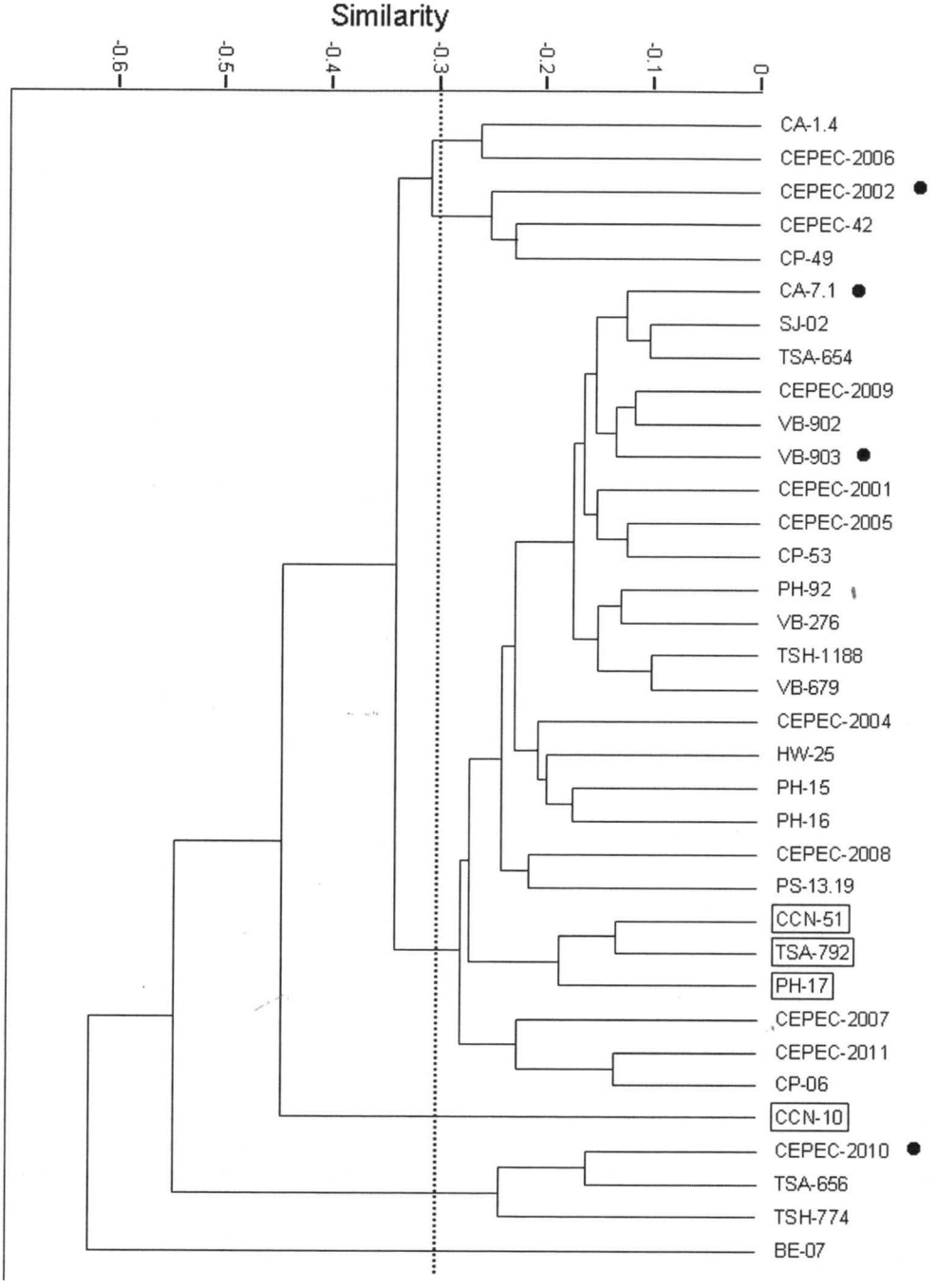

Figure 3. Euclidian distance-based grouping analysis of 35 cacao clones subjected to anoxia, using the ratios between waterlogged and control values for the physiological variables *A*, gs, *E*, Ci/Ca, WUE, *A*/gs, F_o, F_m, F_v/F_m, LB, SB, RB, TB, R/S, NL, LA, ILA and SLB (see Materials and methods). Analysis of similarity was based upon the UPGMA method. The similarity cutoff for definition of groups is indicated by a dashed vertical line. Marked clones on the right showed a similar physiological response to waterlogging, i.e., no chlorosis (boxed) and lowest IS values (black-dotted) after waterlogging treatment (see Table 2 and Figure 2).

and -2010, Table 2) were distributed in three different groups and were closely related to others showing high IS values (>86%). Interestingly though, the four clones with no symptoms of chlorosis (Table 1) constituted two separate groups of one and three clones, respectively (Figure 3). The grouping results obtained by the physiological variables did not resemble those obtained by the analysis of SSR markers (Figures 2 and 3).

Discussion

The rehabilitation program of the cocoa-based economy of southeastern Bahia has been based on the distribution to farmers of elite *T. cacao* genotypes that are more productive and resistant to the witches' broom disease (Souza and Dias 2001). The success of this approach is therefore solely dependent upon an efficient scheme for production and multiplication of plantlets from cacao cuttings, which is negatively affected by conditions causing impairment of their rooting. Waterlogging of the growth medium is caused by periods of heavy rainfall associated with the high levels of moisture kept in the screenhouse (Figure 1); this results in reduced vegetative growth, closure of stomata and decrease in nutrient uptake, which invariably leads to death of plantlets (Schaffer et al. 1992). Preliminary observations showed that cacao genotypes varied in response to anoxia, thereby indicating the need for information that can be useful in the management of mass production of cuttings under screenhouse-fluctuating soil moisture conditions. In this work, relevant information was presented aiming at helping not only with this context but also with further development of selection methodology for high tolerance to waterlogging.

Interclonal genetic variability is essential for development of breeding procedures; a high genetic diversity among the 35 *T. cacao* clones used in this study was confirmed (Figure 2). The clonal genotypes evaluated showed a higher average number of SSR alleles than other studies reporting genetic diversity of cacao based on microsatellites and electrophoresis, either in 3% agarose (Faleiro et al. 2004*a*, 2004*b*) or polyacrylamide gels (Leal 2004). Such a high number of SSR alleles observed here may be explained by the presence of multiple genetic backgrounds of these varieties, generally developed from genotypes of different origins. The superior clones developed in southeastern Bahia were obtained by crossing distinct genetic materials introduced from other countries with elite types identified by local producers (Yamada et al. 2002), which also explains the high levels of heterozygosis observed. Not unexpectedly though, such an interclonal genetic variability was not directly associated with observed patterns of physiological responses to waterlogging, as microsatellites are genomic-based, neutral polymorphic markers not usually associated to any particular phenotype (Hosbino et al. 2002).

Symptoms related to waterlogging stress in tropical fruit trees include leaf chlorosis as well as lenticel and adventitious root formation (Sena Gomes and Kozlowski 1986, Rehem 2006). Leaf chlorosis can be caused by several factors, including accumulation of toxic substances, hormonal dysfunction that leads to senescence, increases in concentration of oxygen-free radicals, lack of adequate nutrition, etc (Pezeshki et al. 1996, Kozlowski 1997, Fageria et al. 2002). Under anoxia, susceptible species generally develop chlorosis as a result of N, P and K deficiencies in the leaves, induced by a decrease in nutrient transport to shoots caused by a reduced root hydraulic conductance (Bingru 2000, Mielke et al. 2003). Studies in woody plants have shown that the development of hypertrophied lenticels at the base of the main stem and at certain parts of submerged roots increases tolerance to flooding (Kozlowski 1997, Drew 1997). These structures are generally associated with aerenchyma in flood-tolerant species, creating mechanisms that allow O_2 influx and diffusion to submerged roots (Topa and Mcleod 1986). The development of lenticels observed in all 35 cacao clones subjected to waterlogging (Table 2) suggests that such an adaptive mechanism is operating in *T. cacao* to cope with anoxia stress. Another observed characteristic related to adaptation to low or null O_2 in the substrate is adventitious root formation, which normally occurs at the base of submerged stem, replacing original roots that died or lost function as a response to waterlogging stress (Vartapetian and Jackson 1997). By providing contact of the plant with air above the flood level, adventitious roots have been deemed to facilitate O_2 uptake and translocation to submerged roots, allowing shoots to maintain growth during prolonged periods of soil flooding (Chen et al. 2002). Considering these traits together, variations in stress responses were observed for the clones, since different combinations of traits and intensities of phenotypic expression could be seen (Table 2). Although no specific phenotype could be directly assigned to a higher or lower survival to waterlogging, it is noteworthy that the absence of chlorosis appeared as a common characteristic of higher IS values (Table 2). This suggests that such a trait may possibly be used as an appropriate morphological marker to trace waterlogging tolerance among genotypes (see further below). Further experiments with a higher number of genotypes displaying such a trait are certainly needed to test this hypothesis.

A rapid decrease in photosynthetic rate following soil flooding has been reported for several angiosperms and gymnosperms, which occurs as a consequence of stomatal closure that decreases CO_2 uptake (Pezeshki 1993; Kozlowski 1997). If the plant stays longer under flooded conditions, a further drop in A occurs as a consequence of direct inhibition of photosynthesis per se. This is strongly associated with degradation of photosynthetic pigments (Pezeshki 2001) as well as with losses in activity of the Calvin's cycle enzymes, such as the ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) (Pezeshki 1994). Some studies state that stomatal closure in flooded plants is mainly due to a decrease in the hydraulic conductance of the roots (Davies and Flore 1986), which raises the internal water stress and reduces leaf turgor and gs values (Pezeshki 2001). In fruit trees, the observed decrease in transpiration rates in response to flooding is likely to occur as a consequence of reduction in gs, since the O_2 deficiency does not significantly decrease the xylem water potential (Schaffer et al. 1992). In woody plants, both tolerant and non-tolerant types decrease gs in response to flooding (Kozlowski 1997), although the former tend to recover to control levels within few weeks after waterlogging (Mielke et al. 2005). An integrative concept is that stomatal reopening is generally related to

the development of hypertrophied lenticels and/or adventitious roots (Lopez and Kursar 1999), which was observed for all genotypes in the present study (Table 2). In terms of photosynthesis, transpiration and stomatal dynamics, the physiological responses to waterlogging were essentially in agreement with these arguments (Table 3). However, the interclonal variations observed did not reveal any specific pattern that could be associated with higher or lower survival rates (Tables 2 and 3).

The biophysical basis of photosynthetic responses can be studied through the fluorescence emission of chlorophyll, in which the F_v/F_m ratio has been considered instrumental to assess the effects of environmental stresses (Maxwell and Johnson 2000, Mielke et al. 2003). The maximal quantum efficiency of PS II is a sensitive indicator of photosynthetic yield, with optimal values around 0.83 for plants of the same species (Björkman and Demmig 1987), as observed for all cacao clones not subjected to anoxia stress. On the other hand, environmental stress can cause the phenomenon of 'photoinhibition', evidenced by higher F_o values and lower F_v/F_m ratios (Maxwell and Johnson 2000), as those noticed for some of the waterlogged plantlets. For the clones displaying statistically significant differences for the variables F_o, F_m and F_v/F_m (Table 3), the major effect of waterlogging was likely upon the photochemical phase of photosynthesis rather than on stomatal closure. This non-stomatal limitation of photosynthesis was specially noticed for the CEPEC-2010, CEPEC-42, TSH-774 and TSA-656 genotypes, which showed the largest waterlogged to control differences for F_v/F_m ratios (Table 3), as well as larger decreases in the A/gs ratio (data not shown). Nevertheless, it is noteworthy that this trait was not associated with rates of survival to waterlogging, since their IS values varied from 54% (CEPEC-2010) to 96% (TSA-656) (Table 2).

Tree species show remarkable decreases in dry biomasses of leaves and roots after 46 days of waterlogging (Ashraf 2003). Anoxia-related reduction in shoot biomass has been attributed to several factors; for instance, the death of roots leads to a decline in the capacity of water and nutrient uptake (Pezeshki and Santos 1998), thereby disturbing the appropriate balance among growth regulators (Carmi and Heue 1981). Decrease in root biomass caused by low metabolic activity and slow growth is also a common response to flooding in trees (Mielke et al. 2005). Changes in root to shoot ratio is also considered an adaptive mechanism to stress by flooding because decrease in biomass allocation to roots reduces metabolic requirements of roots for O_2, water and nutrients (Joly 1994). A similar trend was detected in cacao plantlets, as statistical differences for decrease in root biomass were detected for 17 clones (48.6%), whereas for leaves, only seven clones (20.0%) showed significant decrease in biomass (Table 3). Furthermore, the differences in RB were apparently the major determinants for these changes in R/S ratios, since this variable showed statistical differences between treatments mostly for the same clones (Table 3).

The number of leaves (NL) and leaf area (LA) are two convenient variables whose assessment allows one to investigate altered physiology as a direct consequence of stress-related responses. For instance, anoxia in plantlets of *Betula papyrifera* induced abscission and inhibited the formation of new leaves, with a total decrease of more than 50% of NL in relation to controls after 60 days of flooding (Tang and Kozlowski 1982). Similar responses have been reported for the neotropical tree species *Genipa americana* (Mielke et al. 2003) and *Schinus terebinthifolius* (Mielke et al. 2005), which have also shown modifications in LA. In *Populus* spp., the leaf expansion of flooded plantlets was inhibited by a decrease in cell-wall extensibility (Smith and Bourne 1989). Results obtained for the cutting-derived plantlets of *T. cacao* subjected to 45 days of waterlogging suggest that altered NL and LA responses may not be widely evident in this species, since statistical differences were detected in only five clones (14.3%) for LA, in one clone (2.8%) for NL and in five clones for both traits (Table 3).

A comparative analysis of the results presented in Tables 2 and 3 clearly indicated the existence of interclonal differences for physiological/morphological variables under waterlogged growth conditions; however, statistical differences for these parameters were not necessarily related to their survival rates. Nevertheless, considering such a variety of responses shown by these genotypes and taking a more qualitative rationale into account, a grouping analysis was performed with all variables together, using the ratio between the values obtained from waterlogged and control treatments (Figure 3). The results were striking in suggesting that leaf chlorosis may be phenotypically reflecting how the combined variations of stress responses for the assessed physiological variables are integrated into suitable survival rates for the plantlets. Considering these results together with those indicating the existence of sufficient interclonal genetic diversity (Figure 2), leaf chlorosis may be a valuable parameter to be further investigated as a useful and reliable indicator for the development of breeding strategies toward waterlogging tolerance in the elite cacao materials. Previous studies in other species have already suggested this possibility (Boru et al. 2001, Smethurst and Shabala 2003).

In summary, the 35 cutting-derived plantlets of *T. cacao* clones used in this study revealed a general decrease in growth rates on waterlogged substrate. However, a high interclonal genetic diversity and variation in survival rates and physiological responses to anoxia conditions were also observed. No specific association was found between patterns of physiological responses and tolerance to waterlogging, although the CA-1.4, CEPEC-2009, TSA-654, -656 and -792, CCN-10 and -51 and PH-17 genotypes were considered most tolerant due to their higher IS values and/or their higher average values for most photosynthetic and growth variables assessed. Moreover, the latter four comprise two distinct groups based on similarity analysis of physiological variables and shared the common feature of presenting no symptoms of leaf chlorosis. On the other hand, the CEPEC-2002 and -2010, CA-7.1 and VB-903 were considered the least tolerant

to waterlogging, as their IS values were below 60%. We believe that the results and information presented in this study will not only aid in the establishment of improved strategies for mass production of clonal plantlets of those superior *T. cacao* genotypes but may also be useful to other experimental systems dealing with similar contexts of plant responses to waterlogging in tree species.

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